THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 35, Issue of August 30, pp. 31291–31302, 2002
Printed in U.S.A.

A Genomic and Proteomic Analysis of Activation of the Human Neutrophil by Lipopolysaccharide and Its Mediation by p38 Mitogen-activated Protein Kinase*

Received for publication, January 24, 2002
Published, JBC Papers in Press, April 9, 2002, DOI 10.1074/jbc.M200755200

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Bacterial lipopolysaccharide (LPS) evokes several functional responses in the neutrophil that contribute to innate immunity. Although certain responses, such as adhesion and synthesis of tumor necrosis factor-α, are inhibited by pretreatment with an inhibitor of p38 mitogen-activated protein kinase, others, such as actin assembly, are unaffected. The aim of the present study was to investigate the changes in neutrophil gene transcription and protein expression following lipopolysaccharide exposure and to establish their dependence on p38 signaling. Microarray analysis indicated expression of 13% of the 7070 Affymetrix gene set in nonstimulated neutrophils, and LPS up-regulation of 100 distinct genes, including cytokines and chemokines, signaling molecules, and regulators of transcription. Proteomic analysis yielded a separate list of up-regulated modulators of inflammation, signaling molecules, and cytoskeletal proteins. Poor concordance between mRNA transcript and protein expression changes was noted. Pretreatment with the p38 inhibitor SB203580 attenuated 23% of LPS-regulated genes and 18% of LPS-regulated proteins by ≥40%. This study indicates that p38 plays a selective role in regulation of neutrophil transcripts and proteins following lipopolysaccharide exposure, clarifies that several of the effects of lipopolysaccharide are post-transcriptional and post-translational, and identifies several proteins not previously reported to be involved in the innate immune response.

Lipopolysaccharide (LPS),[1] a component of the outer cell wall of Gram-negative bacteria, evokes a variety of functional responses in the human neutrophil (PMN) after binding to a plasma membrane receptor complex that involves the Toll-like receptors (TLRs) (1–5). These "immediate" functional responses, including actin assembly, adhesion, activation of nuclear factor-kappa B (NF-κB), and priming for an enhanced secretory response and for release of reactive oxygen intermediates, appear to be central both to the innate immune response and to the pathogenesis of several inflammatory human diseases, including sepsis and the acute respiratory distress syndrome (6). p38 mitogen-activated protein kinase (p38 MAPK) has been shown to mediate LPS-induced PMN adhesion, NF-κB activation, and TNF-α and IL-8 translation and release (7), and its blockade attenuates LPS-induced PMN accumulation in the airspace (8). However, other cascades almost certainly lead to downstream effectors of the LPS signal; for example, actin assembly appears to be p38 MAPK-independent (9). An improved understanding of the transcriptional and translational responses of the neutrophil to LPS and the modulation of these responses by p38 MAPK might carry pathogenetic and therapeutic implications.

Historically, it has been believed that the downstream PMN transcriptional response to LPS is static and that PMN functional responses to LPS that depend on *de novo* protein synthesis are primarily limited to the release of cytokines (10). However, recent studies indicate a robust transcriptional response (11). To date, most studies have relied upon and reported a short list of functional assays of the LPS-exposed PMN; therefore, no exhaustive investigation of either the transcriptional response or protein synthetic repertoire of the PMN has been reported. Although several techniques have been used to evaluate transcripts, the screening of global changes in mRNA by microarray analysis has only recently become possible. In this way, thousands of genes can be screened in an unbiased fashion for transcript abundance. Such genomic screens in mammalian cells have previously been applied to define altered expression profiles in response to agonists (12) and to drug action (13) and during cell cycle progression (14).

Although DNA microarray technology is expected to provide insight into the response of the human PMN to LPS (15), inhibition of LPS-stimulated IL-1 and TNF-α production by p38 MAPK inhibitors in THP-1 cells (16) and of TNF-α synthesis in human PMNs (9) occurs at a translational level and would therefore not be detected by DNA microarrays. Furthermore, in other systems, such as yeast and human liver, mRNA and protein levels show poor correlation (17, 18). Proteomics is a complementary tool for assessing global changes in cellular protein expression, thereby providing additional insight into cellular signal regulation. A proteomic approach has proven useful in different systems for dissecting signal transduction cascades and describing their output (19, 20) and has even

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[1] The abbreviations used are: LPS, lipopolysaccharide; DTT, dithiothreitol; IEF, isoelectric focusing; IFN, interferon; IL, interleukin; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; pI, isoelectric point; PMN, neutrophil (polymorphonuclear leukocyte); TLR, Toll-like Receptor; TNF, tumor necrosis factor; CHCA, α-cyano-4-hydroxycinnamic acid; AEBSF, 4-(2-aminoethyl-)benzenesulfonylfluoride hydrochloride; MS, mass spectrometry; CaM, Ca^{2+}/calmodulin; ERK, extracellular signal-regulated kinase; E-64, epoxysuccinyl-64.

recently been used to detect novel upstream messengers involved in LPS signal transduction (21). We have applied DNA microarrays and proteomics to define and compare transcriptional and post-transcriptional alterations in the LPS-exposed PMN and to establish the dependence of these alterations on p38 MAPK signaling.

EXPERIMENTAL PROCEDURES

Materials—Endotoxin-free reagents and plastics were used in all experiments. Aprotinin, leupeptin, AEBSF, E-64, pepstatin, and bestatin protease inhibitors, spermine HCl, and α-cyano-4-hydroxycinnamic acid (CHCA) were all purchased from Sigma Chemical Co. (St. Louis, MO). SB203580, a p38 MAPK inhibitor, was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). For two-dimensional PAGE, rehydration buffer, equilibration buffers, vertical electrophoresis solutions, and 10% homogeneous polyacrylamide slab gels were purchased from Genomic Solutions, Inc. (GSI, Ann Arbor, MI). Sequencing grade porcine trypsin was purchased from Promega (Madison, WI).

LPS Incubation—PMNs were isolated by the plasma Percoll method (22), a technique that yields less than 5% monocytic contamination, and resuspended at a concentration of 15.4×10^6/ml in RPMI 1640 culture medium (BioWhittaker, Walkersville, MD) supplemented with 10 mM HEPES (pH 7.6) and 1% heat-inactivated platelet-poor plasma. After addition of 100 ng/ml *Escherichia coli* 0111:B4 LPS (List Biological), incubation was carried out with continuous rotation (4 h, 37 °C) both in the presence and absence of SB203580. Both Affymetrix analysis and proteomic analysis utilized 75×10^6 cells. For microarray analysis, nonstimulated and 4-h-treated PMNs were collected from three separate donors. A more detailed time course following LPS exposure was performed using polymerase chain reaction. For proteomic analysis, LPS incubations from separate donors ($n = 6$) were performed and then analyzed individually. Control and post-LPS incubation PMNs were washed (0.34 M sucrose/1 mM EDTA/10 mM Tris) and then lysed in a modified rehydration buffer (GSI, Ann Arbor, MI) supplemented with 2 M thiourea, 50 mM dithiothreitol (DTT), 22.5 mM spermine HCl, and a mixture of six protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM AEBSF, 5 μM E-64, 1 μM pepstatin, 10 μM bestatin). DNA was pelleted by centrifugation at 250,000 × *g* for 60 min (23).

Affymetrix Oligonucleotide Array—Five micrograms of total RNA was isolated with TRIzol (Invitrogen) and RNeasy columns (Qiagen) and subsequently labeled with biotin as described by Affymetrix. Briefly, first-strand synthesis was accomplished with Superscript II reverse transcriptase (Invitrogen) using a T7-oligo$(dT)_{24}$ primer for 1 h at 42 °C followed by second-strand synthesis using *E. coli* DNA polymerase I and RNase H (Invitrogen) at 16 °C for 2 h. Double-stranded DNA was used as a template for *in vitro* transcription with T7 RNA polymerase in the presence of biotin-labeled UTP and CTP using the BioArray High Yield RNA transcript labeling kit (Enzo). Fifteen micrograms of cRNA was fragmented and used for hybridization to Affymetrix HuGene 6800FL Genechips. Each sample was hybridized initially using a Test2 Genechip to test for sample degradation and full-length *in vitro* translation. Data were analyzed using Affymetrix Genechip software. Results from three separate donors were analyzed.

Reverse Transcription and Polymerase Chain Reaction—cDNA was prepared by reverse transcription using 2 μg total RNA, derived from 20×10^6 cells that were treated as indicated. Polymerase chain reactions were performed using specific primers for *Mx-1*, *TNF-α*, *MCP-1*, *p65*, *S100A4*, and glyceraldehyde-3-phosphate dehydrogenase.

Two-dimensional PAGE—The protein concentration of the lysates was measured as described by Bradford *et al.* (24). Poor isoelectric focusing (IEF) results were encountered unless the polycationic spermine was diluted (data not shown); therefore, lysates were diluted with rehydration buffer (GSI, Ann Arbor, MI) to achieve a final spermine concentration of 6 mM. Equal protein loads (1.5 mg) of control and LPS-stimulated neutrophils were used to rehydrate IEF gels overnight (18 cm, pH 3–10 nonlinear Immobiline DryStrip IEF gels, Amersham Biosciences; Piscataway, NJ). IEF was performed at 20 °C to 100-kVh (Phaser, GSI) under mineral oil, followed by two 10-min SDS equilibration steps (DTT and then iodoacetamide-containing equilibration buffers, GSI) and then by vertical electrophoresis on 10% homogeneous polyacrylamide slab gels (GSI) at 500 V. Protein spots were visualized by agitation in colloidal Coomassie Brilliant Blue G-250 (16 h) (25), followed by destaining in deionized water (20 h). In separate experiments, control and LPS-stimulated PMN lysates from three donors were pooled and then analyzed by two-dimensional PAGE using overlapping narrow isoelectric point (pI) ranges (18 cm, pH 5.0–6.0, 5.5–6.7, and 6–11, Amersham Biosciences, Piscataway, NJ). Identical IEF and vertical electrophoresis parameters were used for all gels.

Image Analysis of Two-dimensional Gels—Colloidal Coomassie-stained gels were digitized using a Powerlook II (UMAX Data Systems, Inc., Taiwan) flatbed scanner with 8-bit dynamic range and 150-dpi resolution. BioImage (GSI, Ann Arbor, MI) 2D-Analyzer software was used to locate, quantitate, and match protein spots on the control and LPS gel images. Analysis was performed by assigning 50 common anchor spots between paired images; the remaining spots were compared by a constellation-matching algorithm. All data were then carefully reviewed by the operator to account for any discrepancies. Protein loading between control and experimental gels may have varied because of inconsistencies in rehydration of the different IEF gel strips; therefore, gel images were normalized so that the sum of the integrated intensities of all matched spots on paired gels was made equal. Control and LPS-stimulated gel images from individual donor experiments were matched to generate composite images; composite images were then matched into a master composite image to track the LPS response of protein spots among different donors (26). Only those spots that were common (image-matched) to all original 12 (pH 3.0–10.0) gels were considered for further analysis. For these spots, the LPS-induced change in integrated intensity in the six experiments was subjected to statistical analysis with a two-tailed Student's *t* test, and those spots with $p < 0.05$ were identified by peptide mass fingerprinting (described below). For the narrow range (pH 5.0–6.0, 5.5–6.7, and 6–11) two-dimensional PAGE experiments using pooled donors, only those spots with concordant regulation exceeding 1.5-fold or that appeared *de novo* in the LPS gel in two repeat experiments were further analyzed.

In-gel Tryptic Digestion—In-gel digestion of protein spots was performed with sequencing grade porcine-modified trypsin using the method of Hellman *et al.* (27). Tryptic peptides were then extracted (50 μl of 50% acetonitrile/5% trifluoroacetic acid, 2 h), and the supernatant was taken to dryness in a vacuum centrifuge and then redissolved in trifluoroacetic acid (20 μl, 0.5%). Peptides were then purified and concentrated using $ZipTip_{C18}$ pipette tips (Millipore, Bedford, MA).

MALDI-TOF Mass Spectrometry—Analyses were performed on an Applied Biosystems matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager-DE PRO mass spectrometer (Framingham, MA) operated in delayed extraction mode. Samples (0.5 μl) were spotted onto a sample plate to which matrix (0.5 μl of 10 mg/ml CHCA) was added. The sample-matrix mixture was dried at room temperature and then analyzed in reflector mode. CHCA was also spotted alone as a negative control. Spectra were the sum of 100 laser shots, and those peaks with a signal-to-noise ratio of greater than 3:1 were selected for data base searching. Spectra were internally calibrated using autolytic trypsin peptides (*m/z* 842.51, 2211.10).

Data Base Searching Algorithm—The monoisotopic masses for each protonated peptide were: (*a*) entered into the program MS-Fit (available at prospector.ucsf.edu) for searches against the Swiss-Prot, NCBI, and GenPept databases, and (*b*) entered into Mascot (available at matrixscience.com), an algorithm testing statistical significance of peptide mass fingerprinting identifications. For MS-Fit searches, masses derived from trypsin, CHCA, keratin, and Coomassie Brilliant Blue G-250 were excluded. Search parameters included a maximum allowed peptide mass error of 0.1 Da (0.8 Da in the few instances in which linear mode was used), consideration of one incomplete cleavage per peptide, pI range of 3.0–10.0, and molecular mass range of 1–200 kDa. Accepted modifications included carbamidomethylation of cysteine residues (from iodoacetamide exposure following IEF) (28) and methionine oxidation, a common modification occurring during SDS-PAGE (29). Protein identifications were assigned when three criteria were met: 1) statistical significance ($p < 0.05$) of the match when tested by Mascot (matrixscience.com); 2) >20% sequence coverage by the tryptic peptides; and 3) concordance (±15%) with the molecular weight and pI of the parent two-dimensional PAGE protein spot. The following special exceptions were considered: (*a*) protein identifications not fulfilling criterion 2 were still assigned if criteria 1 and 3 were fulfilled and no other *Homo sapiens* proteins with peptide mass-matched *p* values < 0.05 were identified by Mascot; (*b*) if criterion 3 was not fulfilled (lower than expected molecular weight), a cleavage product of the identified protein was inferred, and the cumulative molecular weight of the tryptic peptides was compared with that of the two-dimensional-PAGE spot to ensure that it was not exceeded; (*c*) if criterion 3 was not fulfilled (isolated discordance between theoretical and observed pI), post-translational modification of an unrecovered peptide was inferred; and (*d*) if two or more *H. sapiens* protein assignments with >4 mutually exclusive matching peptides were identified, a protein mixture in the two-dimensional PAGE

spot was inferred and further analysis halted (quantitative conclusions regarding the individual protein constituents could not be drawn).

RESULTS

Genes Differentially Expressed in LPS-stimulated Neutrophils—Human PMNs were left untreated or incubated in the presence of 100 ng/ml LPS for 4 h. As a control to confirm that the PMNs were quiescent at baseline and that LPS resulted in normal stimulation, mRNA was isolated, cDNA was prepared, and PCR for TNF-α was performed. Little TNF-α expression was seen in nonstimulated cells, whereas LPS treatment led to an increase in expression in each of the donors subsequently used for microarray analysis (data not shown). No macrophage-colony stimulating factor receptor transcript was detected by oligonucleotide microarray analysis, confirming there was no significant monocytic contamination.

Human PMNs express a limited repertoire of mRNA transcripts at baseline but respond to LPS with differential expression of genes in many families. Considering only those genes present by microarray analysis in all three donors, unstimulated PMNs expressed 13.0% (923 of 7070 genes) of the Affymetrix gene set. Gene classes represented at baseline include metabolic enzymes, structural proteins, receptors, signaling proteins, and transcription factors. By comparison, human monocytes expressed ~40% and human fibroblasts ~35% of the represented genes (data not shown). By the criterion of a >3-fold increase in expression in all three donors on Affymetrix oligonucleotide array analysis, exposure of PMNs to LPS for 4 h resulted in the up-regulation of 100 genes (Table I).

Genes from several different functional classes were induced in PMNs following LPS exposure. Of interest, a number of transcriptional regulators were induced, including transcription factors of the NF-κB family. The transcriptional NF-κB complex has previously been implicated in the regulation of the genes induced by LPS (11). The genes for several cytokines and chemokines were also found to be up-regulated. These include *TNF-α*, *IL-1β*, *IL-6*, *MCP-1*, *MIP-3α*, and *MIP-1β* (Table I). PCR was performed to confirm the results from the microarray analysis. PCR analysis on selected genes indicates that the time course for changes can be rapid or delayed but parallel the changes found in the array at the 4-h time point (data not shown). Other up-regulated genes included those for metabolic enzymes, immune response molecules, kinases, phosphatases, signaling molecules, adhesion and cytoskeletal components, interferon-stimulated genes, and those with unknown or miscellaneous function (Table I).

LPS stimulation of PMN also resulted in the down-regulation of 56 genes (Table II). Down-regulated genes were identified as transcriptional regulators, protein and lipid kinases and phosphatases, structural molecules, and signaling molecules. Genes for metabolic proteins were also evident, as were several uncharacterized genes.

Two-dimensional PAGE and Image Analysis—In contrast to the limited number of transcripts found at baseline, PMNs were found to express a large number and variety of proteins in the nonstimulated state (Fig. 1, *A* and *C*, and Tables III–V). Reproducible protein expression patterns were found on the pH 3.0–10.0 gels, and the majority of proteins fell in the pH 5.0–7.0 range (Fig. 1*A*). The basic region (pH > 7.0) consistently exhibited poor resolution, precluding meaningful image analysis and further workup (data not shown). Depending on the spot-finding parameters (minimum spot intensity, filter width) selected on the image analysis software, spot-by-spot manual editing was found to be necessary to avoid over- and underdetected spots; moreover, further manual editing was performed to screen for unmatched and mismatched spots following matching of paired control and LPS-stimulated gels. After spot editing, ~1200 well-resolved spots were evident on each pH 3.0–10.0 gel. In an attempt to improve resolution of the pI range bearing the greatest number of well-resolved spots, overlapping narrow pH range gels (pH 5.0–6.0, 5.5–6.7, 6–11) were also run. Of interest, a similar number of well-resolved spots (~1200) were detected on the narrow pH range gels (Fig. 1, *C* and *D*). Assuming a detection limit for Coomassie of 15 ng (0.25 pmol, or 1.5×10^{11} molecules, for a 60-kDa protein) and a protein load per gel corresponding to 75×10^6 PMNs, we estimate a detection limit on our gels of 2000 molecules/cell for a 60-kDa protein. As investigators have suggested in other cell lines with the use of high resolution two-dimensional-PAGE methods (30), we estimate that >10,000 proteins are expressed in the resting PMN.

Human PMNs respond to LPS with the differential expression of a large number of proteins. In the six individual pH 3.0–10.0 experiments, the number of protein spots that increased in integrated intensity by at least 50% following LPS exposure was 185, 122, 104, 104, 96, and 131, respectively. The number of protein spots that decreased by at least 50% following LPS exposure was 72, 151, 102, 98, 128, and 97, respectively. Although gel-to-gel regional variability in resolution was expected to account for individual spots not being well visualized on particular gels, only those spots that were matched to all 12 original gels were analyzed further. Overall, the number of spots matched to all 12 original gels was 125. The numbers of spots that were both matched to all 12 original gels and that increased by at least 50% in integrated intensity in the individual experiments following LPS exposure were 46, 13, 17, 27, 22, and 20, respectively. The numbers of spots that were matched to all 12 gels and that decreased by at least 50% were 6, 22, 17, 22, 34, and 28, respectively. The LPS-induced change in integrated intensity of the 125 spots that were matched to all 12 original gels was subjected to statistical analysis with a two-tailed Student's *t* test, and those spots with statistically significant ($p < 0.05$) regulation among the six experiments were identified by peptide mass fingerprinting (Table III).

Identification of LPS-regulated Proteins—Several proteins were consistently up-regulated on the pH 3.0–10.0 gels (Table III), including regulators of inflammation (annexin III) and signaling molecules (Rab-GDP dissociation inhibitor β). Several actin fragments were seen to be consistently up-regulated in the six experiments following LPS exposure (Table III). Of interest, the proteasome β chain was also consistently up-regulated. Down-regulated proteins included other signaling molecules, such as Rho GTPase activating protein 1.

On the pH 5.0–6.0 and 5.5–6.7 gels, several proteins were found to show increases of greater than 1.5-fold following LPS exposure (Tables IV and V), including cytoskeletal proteins, such as moesin, nonmuscle myosin heavy chain, and a putative phosphorylated form of nonmuscle myosin heavy chain, and signaling molecules, such as protein phosphatase 1 and PO_4-stathmin. The putative phosphorylated form of nonmuscle myosin heavy chain (*spot #1101*) was positioned 0.03 pH unit more acidic than the unmodified protein (*spot #1102*) (Fig. 1*D*) and was distinguished by a tryptic peptide (*m/z* 1366.74) not present in the unmodified protein, consistent with phosphorylation of serine 685. Serine 685 is predicted by NetPhos 2.0 Prediction Server (available at www.cbs.dtu.dk/services/NetPhos/(31)) to be a high probability phosphorylation residue and by ScanProsite (www.expasy.ch/tools/scnpsite.html) to be a substrate for protein kinase C. The tryptic phosphopeptide identified in PO_4-stathmin, extending from residues 15 to 27 (1468.7 Da), is consistent with phosphorylation of either serine 16, a known substrate for Ca^{2+}/calmodulin (CaM)-dependent kinases (32), or serine 25, a known substrate for p38δ and ERK (Fig. 2*A*)

TABLE I
Human neutrophil genes induced after 4 h of LPS exposure

Description	GenBank™ no.	Change-fold
Transcriptional regulation		
Pleiomorphic adenoma gene-like 2	D83784	16.8
NFKB2	S76638	12.3
NFKBIE	U91616	11.5
p65	L19067	8.4
BCL3	U05681	7.7
X-box binding protein 1	M31627	7.5
Metal-regulatory transcription factor 1	X78710	7.4
Ets-2	J04102	7.4
c-Rel	X75042	6.2
NFKB1	M58603	5.8
Basic leucine zipper transcription factor, ATF-like	U15460	4.7
IKB	M69043	3.8
MAX dimerization protein	L06895	3.6
DIF2	S81914	3.1
Cytokines and receptors		
MCP-1	M69203	78.7
MIP-1β	M72885	48.8
αHelix coiled-coil rod homolog	AF014958	20.8
IL-1β	X04500	17.6
GRO3 (beta)	M57731	17.3
TNF-α	X02910	14.5
MIP-3α	U64197	8.1
IL10RA	U00672	7.3
IL-6	Y00081	6.3
GROα	X54489	4
HM74	D10923	3.8
Immune response		
Orosomucoid	X02544	20.2
Complement component *C3*	K02765	12.8
Protease inhibitor 9	U71364	9.5
Complement component 3a receptor 1	U28488	6.1
Protease inhibitor 3	L10343	4.9
SLPI/antileukoprotease	X04470	4.7
ELANH2/elastase inhibitor	M93056	4.6
CD58	Y00636	3.8
Complement component *PFC*	M83652	3.5
Kinases		
CNK/FNK/PLK-like	U56998	16.2
Cot	D14497	11.9
Pim-2	U77735	9.5
LIMK2	D45906	4.3
Phosphatases		
PAC-1/DUSP2	L11329	11.8
DUSP5	U15932	5.3
PHA1	U73477	3.4
Signaling molecules		
TNFAIP1/A20	M59465	10
TRAF1	U19261	6.2
RanBP2	D42063	5.6
GNA15	M63904	5.2
PTAFR	D10202	3.9
Adhesion and cytoskeleton		
ICAM1	M24283	22.4
CEACAM1 (bilary glycoprotein)	X16354	6.3
LIMS1	U09284	6.1
SNL/actin bundling protein	U03057	5.9
Galectin-1/LGALS1	M57710	4.7
MEMD/ALCAM	U30999	4.2
CD44	HG2981—HT3125	3.9
TSG-6	M31165	3.7
Metabolic		
GTP cyclohydrolase I	U19523	13.5
NDUFV2/ubiquinone reductase	M22538	8.6
PSMA6/(proteosome iota)	X59417	8.4
UDP-galactose transporter (*SLC35A2*)	D84454	7.3
PLAU (urokinase)	X02419	6.4
KYNU/L-kynurenine hydrolase	U57721	5.5
AMPD3	D12775	5
P4HA1/prolyl 4-hydroxylase	M24486	4.7
γ Glutamylcysteine synthetase	L35546	4.5
ATP6D	J05682	4.2
ATP6S1	D16469	4

TABLE I—*continued*

Description	GenBank™ no.	Change-fold
Glycerol kinase	X68285	3.6
FACL1	L09229	3.5
AK3	X60673	3.3
Interferon-inducible		
ISG15	M13755	22.5
Mx1	M33882	19.4
IFI56	M24594	12.1
INDO	M34455	5.2
GBPI	M55542	4.3
PRKR	U50648	3.7
IFIT4	U52513	3.6
IFI54	M14660	3.5
IFI58	U34605	3.5
IFP35	U72882	3
Other		
Gos2	M72885	48.8
MIHC/IAP1	U37546	7.2
KIAA0105	D14661	5.1
KIAA0118	D42087	5
SNAP23	U55936	5
CASP5	U28015	4.8
KIAA0113	D30755	4.8
KIAA0255	D87444	4.7
Hepatoma-derived GF	D16431	4.7
PTGS2	D28235	4.6
CD48	M37766	4.3
UNC119 homolog	U40998	4.2
KIAA0151	D63485	3.9
Rab1b	XM035660	3.8
Annexin VII	J04543	3.7
KIAA0110	D14811	3.7
Adrenomedullin	D14874	3.7
AIM1	U83115	3.6
KIAA0250	D87437	3.2
P5-1	L06175	3.2
Scavenger receptor expressed by endothelial cells	D63483	3.2
VHL	L15409	3.1

(33). Assuming that no other multiply phosphorylated stathmin species had escaped detection, analysis of the integrated intensities of the PO_4-stathmin and stathmin spots indicates that the percentage of the PO_4 form of total cellular stathmin increased from 11% to 38% with LPS stimulation (Fig. 2*B*). This is similar to a previous report of an increase from <10% to 35–40% of the Ser^{25}-phosphorylated form in Jurkat cells stimulated with anti-CD3 (34).

Effect of SB203580 on LPS-stimulated Gene Expression—Gene expression analysis of PMNs stimulated with LPS indicated that the majority of genes induced by LPS were unaffected by prior treatment of PMN with SB203580. Of the 100 genes up-regulated by LPS, the up-regulation of 23 was inhibited by greater than 40% (Table VI). The majority of these genes affected by SB203580 were inhibited by less than 60%, whereas only six were inhibited by greater than 80%, all of which represent previously identified interferon-stimulated genes. Induction of cytokine genes by LPS, with the exception of *IL-6*, was generally unaffected by SB203580.

Effect of SB203580 on LPS-stimulated Protein Expression—Similar to the effect of SB203580 on LPS-stimulated gene expression, little effect of SB203580 was seen on expression levels for the majority of LPS-regulated proteins (Table VII). Two exceptions are annexin III and α-enolase, for which LPS-stimulated expression was attenuated in the presence of the p38 MAPK inhibitor.

Comparison of Microarray and Proteomics Results—Of the LPS-regulated proteins identified by peptide mass fingerprinting for which probes were present on the oligonucleotide microarray, poor concordance was found at the mRNA level (Table VIII). For 13 LPS-up-regulated proteins, 2 corresponding mRNA transcripts were up-regulated, 1 was down-regulated, 5 were unchanged, and 5 were not detected by the Affymetrix chip. For 5 down-regulated proteins, 3 corresponding transcripts were down-regulated, 1 was unchanged, and 1 was not detected. Varying patterns of LPS regulation emerge for those candidates detected at both the transcript and protein level. Proteasome β chain was up-regulated at both the transcript and protein levels (Table VIII), with no notable effect of SB203580 on expression at either level. Similarly, CAP1, RhoGAP1, and ficolin 1 were down-regulated at both the mRNA transcript and protein level (Table VIII), with no notable effect of SB203580. Annexin III was down-regulated at the transcript level and up-regulated at the protein level, with an inhibitory effect of SB203580 seen only at the protein level (Tables VII and VIII).

DISCUSSION

Interaction of bacterial LPS with the human PMN represents a model system for studying the activation and output of the innate immune system during infection and inflammation. A recent publication (35) describes the gene expression changes of a cultured monocytic cell line after infection by the Gram-positive bacterium *Listeria monocytogenes*. The cell wall components of Gram-positive bacteria, like Gram-negative-derived LPS (*i.e.* from *E. coli*), are known to signal through TLRs (36, 37). Importantly, many of the expression changes found in LPS-stimulated PMNs in the present study were also described in the bacteria-exposed monocytic cells, indicating that many of the gene expression changes seen in bacterial infection are likely mediated by TLRs (38, 39) and that the LPS model system accurately reflects exposure of immune cells to infec-

TABLE II
Human neutrophil genes repressed (>4-fold) after 4 h of LPS exposure

Description	GenBank™ no.	Change
		-fold
Kinases		
CAMK, II, gamma	U50360	−4
Diacylglycerol kinase, delta	D63479	−4.2
PRKCL2/PRK2 protein kinase C-like 2	U33052	−4.3
MAPKAPK3	U09578	−6.3
Protein kinase Ht31, cAMP-dependent	HG2167–HT2237	−8
CAMK II	L07044	−9.8
Transporters		
SLC25A5/solute carrier family 25, member 5	J02683	−4.2
SLC19A1; folate transporter	U17566	−4.4
SLC2A3; facilitated glucose transporter	M20681	−5
Metabolic		
Carbonic anhydrase IV	L10955	−4.4
RNase A family, k6	U64998	−4.5
Glycogen phosphorylase; liver	M14636	−4.6
Inositol polyphosphate-5-phosphatase	U57650	−4.6
Inositol 1,3,4-trisphosphate 5/6-kinase	U51336	−4.7
Transketolase	L12711	−4.8
Protein phosphatase 4, reg. subunit 1 (clone 23840)	U79267	−4.9
Cytidine deaminase	L27943	−5.4
MGAT1	M55621	−5.4
HMOX1	X06985	−5.4
MAN2A2	L28821	−5.8
Glycogenin (also represents U31525)	HG4334–HT4604	−5.9
Structural		
Fibrinogen-like protein (pT49 protein)	Z36531	−4.2
H2AFZ	M37583	−4.7
Paxillin	U14588	−4.9
Lamin B R	L25931	−5.9
Dynamin 2	L36983	−6.2
Actinin 1	M95178	−6.7
α-Tubulin	X01703	−10
Tubulin, α1, isoform 44	HG2259–HT2348	−15
Transcriptional regulators		
Lymphoblastic leukemia-derived sequence 1	M22638	−4.4
MAX-interacting protein 1	L07648	−4.5
Nuclear factor crythroid 2 isoform f	S77763	−6
Transducer of ERBB2, 1	D38305	−6.9
NFATC4	L41067	−7.8
ATF-2 (CRE-Bpa)	L05515	−9.6
Receptors		
Lymphotoxin β receptor	L04270	−4.4
Folate receptor 3 (gamma)	U08471	−5
	U11875	−5.3
Signaling		
Pix-α; cool-2 (KIAA0006)	D25304	−4.5
ARHB/RhoB	M12174	−4.5
TNFSF10; TRAIL	U37518	−6.6
Ca^{2+} binding		
ANXII	L19605	−4.3
S100A4	M80563	−4.8
ANX1	X05908	−4.8
Other		
Proteolipid protein 2	L09604	−4.9
Protein phosphatase 1, α catalytic subunit	HG1614–HT1614	−5
TIMP2	M32304	−5.1
KIAA0199	D83782	−5.2
Lipin 2 (KIAA0249)	D87436	−5.6
LRMP (Jaw1)	U10485	−5.8
CUGBP2	U69546	−6.9
Clone 23933	U79273	−7
PECAM1	L34657	−8
Delta sleep-inducing peptide	Z50781	−8.7
DiGeorge synd. critical region gene 2 (KIAA0163)	D79985	−9
SELPLG; CD162; selectin P ligand	U25956	−32

tion. Nevertheless, the reliance upon DNA microarrays alone affords insight only into the transcriptional response without corroboration at the protein level. In the present study, application of both DNA microarray and proteomics technology to our model system provides unique insight into both the cellular biology of the activated PMN and the responsiveness and reg-

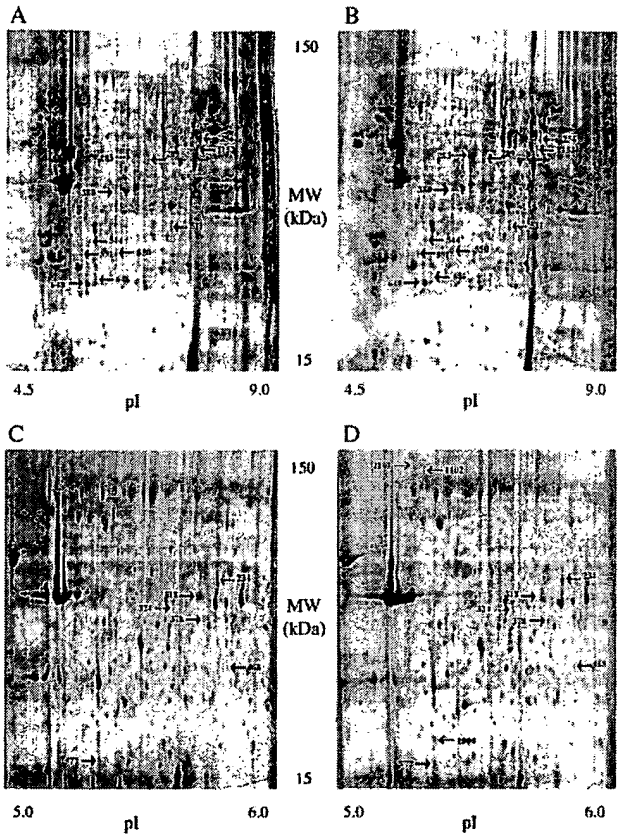

FIG. 1. **Two-dimensional PAGE of LPS-exposed human PMNs.** *A* and *B*, colloidal Coomassie Blue-stained pH 3.0–10.0, two-dimensional PAGE gels (*A*, control; *B*, LPS-exposed) with up-regulated (*solid arrows*) and down-regulated (*hatched arrows*) proteins indicated. These results are representative of six separate experiments. *C* and *D*, colloidal Coomassie Blue-stained pH 5.0–6.0, two-dimensional PAGE gels (*C*, control; *D*, LPS-exposed) with up-regulated (*solid arrows*), new (*solid arrow, open arrowhead*), and down-regulated (*hatched arrows*) proteins indicated. LPS-exposed PMNs from three blood donors were pooled.

ulation of its transcriptional and translational machinery. As will be discussed below, our study identifies, in particular, novel aspects of the LPS-stimulated PMN transcriptional regulation, activity in the innate immune response, signaling, cytoskeletal reorganization, and priming for granule release.

In the present study, the increase in NF-κB transcript abundance (Table I) detected by the microarrays corroborates the findings of other studies of PMNs and monocytes (40) and indicates a mechanism for the responsiveness and scope of the PMN transcriptional machinery following LPS exposure. NF-κB, recently described to be activated by LPS through the TLR/MyD88/interleukin-1 receptor-associated kinase pathway (1, 4), is the only transcriptional complex reported to be induced by LPS in the PMN. However, because the transcriptional NF-κB complex has been implicated in the regulation of only a portion of the genes induced by LPS in this study (data not shown), the importance of alternative transcriptional regulators in the PMN is clear. Of interest, several other known and putative transcriptional regulators with less well defined functions were also up-regulated in the present study, including *PLAGL2*, a putative zinc-finger protein, *XBP-1*, *MTF-1*, *Ets-2*, *B-ATF*, and *DIF-2*. On the other hand, LPS-down-regulated genes include *ATF-2* (a known target of p38), *NFATC4*, *TOB-1*, *NF-E2*, *MXI-1*, and *LYL-1*. Although the exact role of these gene products in regulating cell function is unknown, these data indicate that the range of transcriptional responses in the LPS-stimulated PMN is much broader than previously suggested and that the signaling capabilities of the PMN in the immune response are thereby likely extended in scope and specificity.

As expected from the literature, the genes for several cytokines and chemokines, including *IL-1β*, *IL-6*, and *MIP-1β*, were found to be up-regulated (Table I). On the other hand, the notable absence of up-regulated cytokines in the proteomics experiments reflects their removal in the post-LPS incubation wash performed prior to lysis for two-dimensional-PAGE. Up-regulation of these inflammatory mediators is well documented in PMNs exposed to LPS and in animal models of LPS-induced sepsis syndrome and acute respiratory distress syndrome, a PMN-mediated illness (41, 42). Several genes in this family were up-regulated that have not, to our knowledge, been described in LPS-stimulated cells, including *MCP-1*, *GRO3*, *IL-10RA*, and *HM74*, an orphan G protein-coupled receptor with homology to chemokine receptors. The down-regulation of *TNFSF10*, *lymphotoxin b receptor*, and *TNFAIP1* were also observed. The modulation of genes involved in cytokine signaling, including the adapter molecules *TRAF1* (LPS and TNF receptor signaling) and TNFAIP1 (TNF receptor signaling) and several kinases and phosphatases, may indicate a change in cytokine responsiveness after LPS treatment. Relevant in this regard from the proteomics data are: 1) the up-regulation of protein phosphatase 1, which has been shown to regulate PMN NADPH oxidase activation and translocation (43, 44) and to regulate LPS-induced NF-κB activation (45); 2) the down-regulation of Rho-GAP1, which has been shown to regulate NADPH oxidase activity in the PMN (46); and 3) the up-regulation of PO_4-stathmin (Table IV), a phosphoprotein postulated to function as a relayer and integrator of multiple signal transduction pathways (34). Several noncytokine, nonchemokine genes involved in the immune response were also up-regulated, including the complement pathway members *C3*, *C3AR1*, and *PFC*; the protease inhibitors *ELANH2* (elastase inhibitor), *SLPI*, *PI-3*, and *PI-9*; and the acute phase protein *orosomucoid*. LPS regulation of *C3AR1* and *orosomucoid* expression have not previously been reported. In the proteomics experiments, the down-regulation of ficolin-1 (Table III), a collectin-like cell surface protein reported to activate the complement system and to mediate adhesion and phagocytosis in monocytes but not previously reported in granulocytes (47), may represent negative modulation of the innate immune response. The finding that genes other than cytokines and chemokines are regulated by the PMN in response to LPS indicates that the PMN plays a more sophisticated role in host-defense and immunity than previously thought.

Treatment of the PMN with LPS lead to the induction of a set of genes associated with the anti-viral Type I interferons, IFNα/β. This induction occurs independently of the release of IFN or another unidentified soluble factor.[2] Furthermore, the set of genes expressed is smaller than that induced by IFNα/β, as described by Der *et al.* (12). This may be due to differences in the scope of the signaling systems activated by LPS and IFNα/β, or the time course of analysis of genes in the LPS-stimulated PMN. The implication that LPS treatment of PMN allows PMN to express anti-viral activity is currently being tested. Of interest was the finding that induction of interferon-stimulated genes was blocked by pretreatment of PMNs with SB203580. Work from our laboratory has indicated that signal transducers and activators of transcription activation does not occur in response to LPS in PMNs.[2] In addition, interferon-

[2] K. C. Malcolm and G. S. Worthen, manuscript in preparation.

TABLE III
Analysis of pH 3.0–10.0 two-dimensional PAGE gels

Mean change(-fold) in expression level among six PMN donors is reported. The change in expression for the proteins listed was statistically significant ($p < 0.05$) as measured by a two-tailed Student's t test.

Identification [spot no.]	Swiss-Prot no.	Estimated M_R/pI	Theoretical M_R/pI	Peptides matched/ submitted	Protein covered	Mean change
				%	%	*-fold*
Up-regulated						
Proteasome β chain [646]	P28070	27/5.7	29.2/5.72	9/12 (75%)	36%	1.51
Annexin III [550]	P12429	31/5.7	36.4/5.6	14/18 (78%)	42%	1.37
Actin *fragment* [544][a]	P02570	32/5.5	(41.7/5.29)	13/15 (87%)	(34%)	1.74
Actin *fragment* [591][a]	P02570	30/5.4	(41.7/5.29)	14/18 (78%)	(29%)	1.60
α-Enolase [380]	P06733	41/5.7	47.2/7.01	9/10 (90%)	24%	1.65
Rab-GDP dissociation inhibitor β [289]	P50395	50/6.1	50.7/6.11	10/11 (91%)	25%	1.24
Glutathione *S*-transferase P [648]	P09211	23/5.5	23.4/5.43	6/8 (75%)	41%	1.54
Pre-B-cell colony enhancing factor [1152]	P43490	53/7.0	55.5/6.69	12/16 (75%)	25%	1.29
Down-regulated						
Adenylyl cyclase-associated protein 1 [256]	Q01518	55/7.3	51.7/8.07	16/22 (73%)	34%	0.53
Rho-GAP1 [283]	Q07960	50/5.8	50.4/5.85	7/9 (78%)	22%	0.67
Ficolin 1 [511]	O00602	33/6.5	35/6.39	10/12 (83%)	25%	0.74

[a] The theoretical pI and M_R of native actin are indicated. Protein coverage indicates coverage of native actin.

TABLE IV
Analysis of pH 5.0–6.0 two-dimensional PAGE gels

Results are from pooled samples for control ($n=3$) and LPS-exposed ($n = 3$) PMNs from human donors. Expression of the reported proteins was altered >1.5-fold following LPS exposure in two repeat experiments. "New" designates proteins seen in the LPS gel in two repeat experiments but not detectable in the corresponding control gels.

Identification [spot no.]	Swiss-Prot no.	Estimated M_R/pI	Theoretical M_R/pI	Peptides matched/ submitted	Protein covered	Change
				%	%	*-fold*
Up-regulated						
Protein-tyrosine kinase 9-like [468]	Q9Y3F5[a]	34/5.81	39.5/6.37	10/14 (71%)	34%	1.8
Protein phosphatase 1, catalytic subunit, β isoform [378]	P37140	38/5.73	37.2/5.84	7/10 (70%)	22%	2.0
PO_4-stathmin [577]	P16949[b]	18/5.36	17.3/5.76	9/12 (75%)	42%	2.1[e]
Nonmuscle myosin heavy chain [1102]	189036[c]	145/5.32	145/5.23	20/21 (95%)	17%	New
Putative PO_4-nonmuscle myosin heavy chain [1101][d]	189036[b,c]	145/5.29	145/5.23	14/16 (87%)	13%	New
Leukocyte elastase inhibitor [318]	P30740	42/5.71	42.7/5.9	9/13 (69%)	22%	2.4
Grancalcin [1004]	P28676	24/5.36	24.0/5.02	7/10 (70%)	31%	New
Down-regulated						
Adenosylhomocysteinase [324]	P23526	48/5.82	47.7/6.04	7/9 (78%)	14%	0.4
PEST phosphatase interacting protein homolog [234][g]	4100162[f]	48/5.30	47.6/5.35	11/13 (85%)	30%	0.5

[a] TrEMBL accession number.
[b] Accession number and theoretical pI and M_R for the unmodified protein are indicated.
[c] *N*CBI accession number.
[d] See text for explanation.
[e] Among three experiments, the ratio of PO_4-stathmin expression increase, following LPS exposure in the presence of SB203580 divided by that in the absence of SB203580, was 0.93.
[f] Genpept accession number.
[g] This search was performed using average masses measured by linear mode MALDI-TOF MS.

TABLE V
Analysis of pH 5.5–6.7 two-dimensional PAGE gels

Results are from pooled samples for control ($n = 3$) and LPS-exposed ($n = 3$) PMNs from human donors. Expression of the reported proteins was altered >1.5-fold following LPS exposure in two repeat experiments.

Identification [spot no.]	Swiss-Prot no.	Estimated M_R/pI	Theoretical M_R/pI	Peptides matched/ submitted	Protein covered	Change
				%	%	*-fold*
Up-regulated						
Transaldolase [475]	P37837	38/5.95	37.5/6.36	13/17 (76%)	33%	2.5
Isocitrate dehydrogenase [431]	O75874	46/6.25	46.7/6.35	7/7 (100%)	13%	2.3
Moesin [201]	P26038	61/6.09	67.8/6.07	11/13 (85%)	17%	2.1
α-Enolase [459]	P06733	43/5.64	47.2/7.01	7/10 (70%)	17%	3.8
Down-regulated						
Calponin H2 [240]	Q99439	34/6.65	33.7/6.94	10/11 (90%)	27%	0.5

regulatory factor 3, a known regulator of interferon-stimulated gene transcription, is not a direct target of p38 kinase.[2] Therefore, gene expression analysis of LPS-stimulated PMNs has uncovered a previously uncharacterized signal transduction system that is sensitive to inhibition of p38 MAPK.

Knowledge of the genes down-regulated by LPS permits the development of further hypotheses addressing PMN function in the face of infection. Strikingly, several down-regulated genes and gene products are structural in nature (*e.g.* paxillin, actinin, calponin H2) (Tables II and V). A known consequence to the PMN of LPS exposure is decreased motility (48). Up-regulation of genes for adhesion molecules (*ICAM-1*, *CD44*, *AL-*

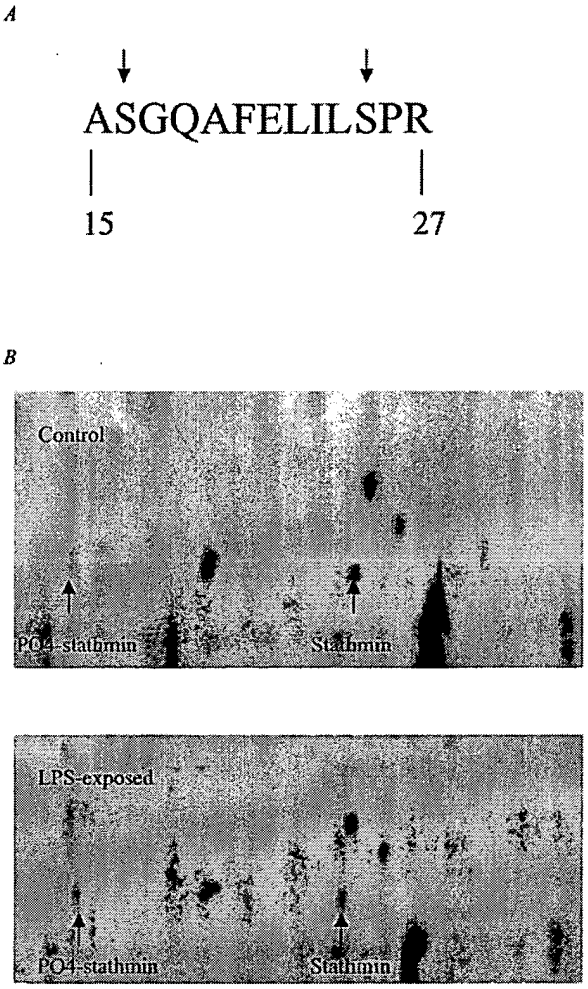

FIG. 2. *A*, the predicted sequence of the tryptic phosphopeptide in PO_4-stathmin (1468.72 Da). The peptide mass measured by MALDI-TOF MS and the predicted mass differed by 14 ppm. As indicated, two alternate phosphorylation sites are possible: serine 16 and serine 25. *B*, PO_4-stathmin and stathmin were identified on the control and LPS-exposed pH 5.0–6.0 gels. Consistent with phosphorylation, the PO_4-stathmin spot was distinguished by a peptide of mass 1468.72 Da (*i.e.* 80 Da greater than the peptide of 1388.72 Da seen in the stathmin spot). Assuming that no other multiply phosphorylated stathmin species have escaped detection, analysis of the integrated intensities of the PO_4-stathmin and stathmin spots indicates that the percentage of the PO_4 form of total cellular stathmin has increased from 11% to 38% with LPS stimulation. The decrease in integrated intensity for stathmin was equal in amount to the increase in PO_4-stathmin following LPS exposure.

CAM, and *TSG-6*), and down-regulation of genes for structural proteins, indicates a genetic basis for this observation. Down-regulation of two genes implicated in cytoskeletal regulation, *Pix-α* and *RhoB*, was also observed. The calcium-binding protein S100A4, down-regulated in LPS-treated PMNs (Table II), has been implicated in cell motility and metastasis (49). Decreased motility may be beneficial in sustaining the inflammatory response at sites of infection. In addition, LPS treatment results in an inhibition of apoptosis (50). Therefore, the longer residence time of the PMN at sites of infection is consistent with the long term genetically coded changes seen in these gene-profiling experiments and indicates that the changes in gene expression are functionally relevant to host defense and immunity.

By providing information on post-translational modification, the proteomics data may provide further insights into the cytoskeletal remodeling effects of LPS upon the PMN. We contend that the actin fragments identified (Table III) are unlikely to represent technical artifacts. Rather, their specificity (identical molecular weight/pI among different experiments), statistically significant up-regulation by LPS, as well as the use of a lysis buffer containing chaotropes and multiple protease inhibitors argue instead that these fragments are physiologic consequences of LPS exposure in the human PMN. More specifically, the up-regulation of these fragments following LPS exposure (Table III) suggests that LPS may activate an actin-cleaving enzyme, which, in turn, remodels the cytoskeleton. Intriguing in this vein, calpain has recently been reported to play an important role in cell migration and cytoskeletal organization of fibroblasts (51). The possibilities that LPS may induce calpain activation and that calpain activation may regulate cytoskeletal reorganization and motility are currently under investigation. An alternative possibility is that actin cleavage is a marker of neutrophil apoptosis (52).

Other LPS-regulated proteins may play important roles in cytoskeletal reorganization. The up-regulation of protein-tyrosine kinase 9-like (A6-related protein) may modulate LPS-

TABLE VI
Effect of SB203580 on LPS-stimulated gene expression

Genes are reported for which the SB203580/control expression ratio is ≤ 0.60.

Gene name	-fold change ratio (SB203580/control)	Change in absence of SB203580
		-fold
ISG15	0.09	22.5
HCR	0.38	20.8
Mx-1	0	19.4
IFI56	0	12.1
PI-9	0.57	9.5
Ets-2	0.59	7.4
IL-6	0.45	6.3
Rel	0.50	6.2
LIMS1	0.58	6.1
C3AR1	0.49	6.1
INDO	0.35	5.2
KIAA0105	0.41	5.1
SNAP23	0.58	5.0
SLPI	0.58	4.7
ELNAH2	0.49	4.6
HM-74	0.57	3.8
PKR	0	3.7
MAD	0.21	3.6
IFIT4	0.12	3.6
Glycerol kinase	0	3.6
IFI54	0	3.5
IFI58	0.39	3.5
IPF35	0.46	3.0

TABLE VII
Effect of SB203580 on LPS-stimulated protein expression

Protein name	-fold change ratio (SB203580/control)	Change in absence of SB203580
		-fold
Up-regulated		
Proteasome β chain	0.8	1.51
Annexin III	0.6	1.37
Actin *fragment* [544]	0.8	1.74
Actin *fragment* [591]	0.8	1.60
α-Enolase	0.6	1.65
Rab-GDP dissociation inhibitor β	1.1	1.24
Glutathione *S*-transferase P	1.2	1.54
Pre-B-cell colony enhancing factor	1.2	1.29
Down-regulated		
Adenylyl cyclase-associated protein 1	1.3	0.53
Rho-GAP1	0.8	0.67
Ficolin 1	1.0	0.74

TABLE VIII
LPS-regulated proteins for which a probe was present on the Affymetrix chip

A comparison of corresponding protein and mRNA transcript changes following LPS exposure is shown.

Protein	Protein change	mRNA change
		-fold
Up-regulated		
Proteasome β chain	1.5	1.9 ↑
Leukocyte elastase inhibitor	2.4	4.6 ↑
Rab-GDI β	1.24	NC[a]
Grancalcin	New	NC
Transaldolase	2.5	NC
Moesin	2.1	NC
Nonmuscle myosin heavy chain	New	NC
Glutathione *S*-transferase P	1.54	Absent
Pre-B cell enhancing factor	1.29	Absent
Isocitrate dehydrogenase	2.3	Absent
PO_4-stathmin	2.1	Absent (stathmin)
Protein phosphatase 1, β catalytic subunit	2	Absent
Annexin III	3.1	3.1 ↓
Down-regulated		
Adenylyl cyclase-associated protein 1	1.9	2.1 ↓
Rho-GAP 1	1.5	2.7 ↓
Ficolin 1	1.4	1.7 ↓
Adenosylhomocysteinase	2.5	Absent
Calponin H2	2	NC

[a] NC, no measureable change.

induced actin polymerization, because it bears a high degree of homology to twinfilin (A6), an actin monomer-binding protein that localizes to sites of rapid filament assembly in cells and is believed to regulate actin filament turnover (53). In turn, LPS-induced down-regulation of Rho-GTPase activating protein 1 (Table III) may regulate twinfilin (and protein-tyrosine kinase 9-like) activity, because twinfilin has been shown to colocalize with Rac1 and Cdc42 and to be regulated by active Rac1 in NIH 3T3 cells (53). Activation of Rho proteins may be facilitated by LPS up-regulation of moesin (Table V), because moesin reportedly induces the dissociation of Rho from GDI (54). Rac1 may, in turn, promote activation of the actin filament-nucleating Arp2/3 complex through interactions with WASP (Wiskott-Aldrich syndrome protein) family proteins (55) and, interestingly, is postulated to regulate the dynamics of both the actin and microtubule cytoskeletons via phosphorylation of stathmin (Table IV) (56). Calponin H2 is an actin-binding protein not previously reported in PMNs that is postulated to play a role in cytoskeletal organization (57). Its down-regulation by LPS (Table V) likely modulates LPS-induced cytoskeletal reorganization. The up-regulation of nonmuscle myosin heavy chain and a putative phosphorylated form of myosin heavy chain (putative protein kinase C substrate by prediction rules) in the LPS-exposed PMN (Table IV) is of uncertain significance; myosin has been implicated in multiple functions in the PMN, including locomotion, fluid pinocytosis, and phagocytosis (58). Of interest, however, S100A4 (down-regulated, Table II) has been reported to regulate cytoskeletal dynamics by inhibiting protein kinase C-mediated phosphorylation of nonmuscle myosin heavy chain (59).

LPS induction of stathmin phosphorylation (Table IV and Fig. 2) may represent another mechanism by which the cytoskeleton is remodeled. Stathmin is a phosphoprotein reportedly involved in both signal transduction and in regulation of the microtubulin filament network; furthermore, phosphorylation of stathmin has been reported to modulate its tubulin-binding avidity (60). Inferences can be made about both the phosphorylation site on PO_4-stathmin and the responsible kinase induced by LPS. Four phosphorylation sites in stathmin have been well described: Ser^{16}, Ser^{25}, Ser^{38}, and Ser^{63} (32, 33). Ser^{16} has been reported as a substrate for Ca^{2+}/calmodulin (CaM)-dependent kinases (32), and Ser^{25} as primarily a substrate for p38 and ERK (33), with $p34^{cdc2}$ also active but bearing a 5-fold preference for Ser^{38} (34). As stated above, the phosphopeptide identified in PO_4-stathmin, extending from residues 15 to 27 (1468.7 Da), is consistent with phosphorylation of either Ser^{16} or Ser^{25} (Fig. 2). Although both p38δ and p38α MAPK isoforms are expressed in the human PMN, LPS has been shown to selectively activate the p38α isoform in human PMNs (9). The p38α isoform, however, has been shown to be relatively inactive at Ser^{25}; in fact, p38δ is ~100-fold more active at Ser^{25}, and selective p38α inhibitors do not inhibit the stress-activated phosphorylation of stathmin in 293 cells (33). Further support for the lack of involvement of p38 signaling in phosphorylation of stathmin in our system is the apparent lack of effect of SB203580 (a selective p38α and p38β inhibitor) on LPS-induced expression of PO_4-stathmin (Table IV). Because $p34^{cdc2}$ is relatively inactive at Ser^{25} (34), we conclude that the phosphorylation site is likely to be Ser^{16}, a reported substrate of CaM-dependent kinase. Although CaM kinases have previously been implicated in gene activation in LPS-exposed myelomonocytic HD11 cells (61), stathmin signaling has not, to our knowledge, been previously reported in either PMNs or lipopolysaccharide signal transduction.

Cytoskeletal reorganization, a well-described regulator of granule release (62), may underlie LPS-induced priming for PMN granule release, but several LPS-regulated proteins may provide more specific clues. LPS exposure led to increased levels of grancalcin, a calcium-binding protein previously detected in PMNs and shown to translocate to granules and plasma membrane in the presence of physiologic concentrations of calcium (63). Similarly, annexin III, a calcium-binding protein highly expressed in PMN granule membranes and implicated in calcium-mediated secretion (64) and in granule fusion (65), was also found to be up-regulated. Exocytosis of granule contents may also be facilitated by LPS up-regulation of Rab-GDP dissociation inhibitor (Table III), which has been proposed to recycle Rab after vesicle fusion by extracting it from the membrane and loading it onto newly formed transport intermediates (66).

Parallel use of DNA microarrays and proteomics affords a powerful strategy for comparison of corresponding mRNA transcripts and proteins, thereby affording new insight into the mechanisms by which the cell regulates its signaling responses to the external environment. Of interest, a poor correlation was found between corresponding transcripts and proteins (Table VIII), as reported in other systems (17, 18). The finding in some cases of unchanged transcript abundance in the face of regulated protein levels indicates post-transcriptional modulation following LPS exposure. The finding of undetected transcripts in the face of regulated levels of the corresponding proteins may indicate previous transcription of these genes in an earlier state of the myeloid maturation of the PMN, producing stable protein species that have undergone post-translational alteration following LPS exposure. The use of SB203580, a p38 inhibitor, adds further insights into the mechanisms of LPS regulation. At the level of mRNA expression, SB203580 inhibited 23% of LPS-stimulated genes by ≥40% and 11% of genes by ≥60%; therefore, p38 plays a specific role in gene regulation in the PMN. In particular, proteasome β chain was up-regulated at both the mRNA transcript and protein level (Table VIII), with no notable effect of SB203580 on expression at either level, consistent with a non-p38-mediated pathway of primary transcriptional up-regulation induced by LPS. Similarly, CAP1, Rho-GAP1, and ficolin 1 were down-regulated at both the mRNA transcript and protein level (Table VIII), with

no notable effect of SB203580, consistent with a non-p38-mediated pathway of primary transcriptional down-regulation. Interestingly, annexin III was down-regulated at the transcript level and up-regulated at the protein level, with an inhibitory effect of SB203580 seen only at the protein level (Table VII), consistent with a p38-mediated post-transcriptional up-regulation induced by LPS.

Limitations of the present study should be noted. Gene expression analysis by cDNA microarrays does not distinguish between transcriptional regulation and mRNA stabilization; similarly, two-dimensional PAGE proteomics by itself does not distinguish among transcriptional, translational, or post-translational regulation of protein abundance. Transcript detection by microarray technology is limited to the probes included; protein identification by two-dimensional PAGE proteomics is limited to well-resolved regions of the gel, may perform less well with hydrophobic and high molecular weight proteins, and tends to select for more abundant protein species (30). Harvesting of the LPS-incubated PMNs at 4 h may have prevented detection of earlier, transient changes and may have thereby introduced artifactual transcript-protein discordance. Furthermore, the post-LPS incubation, pre-two-dimensional PAGE cell washes would be expected to remove secreted proteins from further analysis, with uncertain effects on detected protein abundance depending on such factors as the degree of *de novo* synthesis and extent of degranulation/exocytosis. Because protein binding of Coomassie Blue has a limited dynamic range and is typically not linear throughout the range of detection, image analysis of Coomassie Blue-stained protein spots should be considered semi-quantitative. For some protein spots, the apparent magnitude of regulation by LPS may have been blunted by the spot approaching staining saturation in the control gel. By limiting our analysis to those protein spots common to all twelve pH 3.0–10.0 two-dimensional gels, we likely excluded some LPS-regulated proteins that happened to be either poorly resolved on a subset of the gels or unmatched by the image analysis software. By further limiting the analysis to those matched spots on the pH 3.0–10.0 gels for which a two-tailed t test demonstrated $p < 0.05$, the list of regulated proteins was likely also limited by statistical power. In addition to those regulated proteins listed in Table III, three others were up-regulated and three down-regulated with $p < 0.09$ (data not shown).

Limiting our reported results to those changes that met statistical significance among the donors carries further important implications. We have encountered a two order of magnitude range of response in unselected donor LPS-induced PMN functions, such as TNF-α and superoxide anion release (data not shown). The sources of this physiologic heterogeneity remain uncertain but may possibly include such factors as natural mutations of the LPS receptor component, TLR4 (67). By selecting for LPS effects common to all donors, we may not have characterized the range of genomic and proteomic heterogeneity present in the population and thereby may have focused on only a narrow portion of a broader biological response to LPS. We contend that this reductionist approach is valid because it would be expected to enrich for biologically integral responses of the PMN to LPS. Nevertheless, correlation of genomic and proteomic profiles with functional phenotypes of the PMN may bear important diagnostic and therapeutic implications and will be pursued in future studies.

Widespread regulation of numerous noncytokine/chemokine genes and proteins in the LPS-stimulated human PMN is a novel finding. These data indicate that, despite a narrow scope of gene expression in the nonstimulated state, the terminally differentiated, short-lived PMN likely plays a role in the innate immune response that is far more sophisticated and dynamic than the simple release of preformed inflammatory mediators. Although gene expression appears to be an important mechanism by which PMNs respond acutely to infection, mRNA transcript/protein concordance is limited, and post-transcriptional (and post-translational) modifications also play an important role. The alteration of multiple transcriptional regulators, G-protein regulators, PO_4-stathmin, and protein phosphatase 1 indicates that one of the responses to LPS exposure is to modify subsequent signaling events by bacterial components or by other cytokines and chemokines. Finally, the finding that p38 MAPK mediates LPS regulation of a limited subset of transcripts and proteins underlines the continuing need to define signal transduction cascades in the neutrophil.

Acknowledgments—We thank the members of the Affymetrix core laboratory, University of Colorado Health Sciences Center, as well as Benjamin Perryman, Steve Helmke, and Jennifer Lynch of the Cardiology Division, University of Colorado Health Sciences Center for assistance with two-dimensional PAGE.

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